

STUDIES IN THE ENZYME MAKE-UP OF *ALTERNARIA*

I. Qualitative Demonstration of Enzymes

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ABSTRACT

Experiments were conducted with three phytopathogenic species of *Alternaria* to find out their enzyme make-up. The presence of a large number of enzymes has been demonstrated in the three species. Phosphorylase, aldolase, dehydrogenases, amidases, deaminases, ribonuclease, nucleodeaminases, glycerophosphatase and nucleophosphatases have been shown to be present in *Alternaria* for the first time.

A LIMITED aspect of enzyme system of *Alternaria* has received attention from previous workers (Szelenyi and Beeze, 1928; Uppal *et al.* 1938; Tandon and Srivastava, 1949; Coulson and Cinq-Mars, 1952; Torikata and Komai, 1952; Franke and Taha, 1952; Ozawa, 1952; Ozawa and Okamoto, 1953; Pomeranz, 1957; Meyers and Reynolds, 1959). The present series of investigations were undertaken with a view to elucidate the enzyme make-up of three authenticated species of *Alternaria*, viz., *A. tenuis* auct. sensu Wilshire (AT), *A. brassicola* (Schw.) Wilshire (AB) and *A. raphani* Groves and Skolko (AR), isolated from their respective hosts *Althea rosea*, *Brassica oleracea* and *Raphanus sativus*. Of these *A. tenuis* has already been worked out (Tandon and Srivastava, 1949; Coulson and Cinq-Mars, 1952; Pomeranz, 1957; Meyers and Reynolds, 1959) though only briefly, whereas the other two species are metabolically unknown. The underlying idea behind these studies was to find out the possible correlation between the enzyme make-up of these strains and their pathogenicity. In this first paper of the series an account is given of the enzymes detected by means of qualitative procedures.

Pure monohyphal cultures of these species were utilized for investigation and periodically tested for purity. For the preparation of extra- and intracellular enzyme extracts, mycelia from young cultures (Brown, 1915) were utilized, as being the stage when maximum enzyme production is known to occur. The medium used to grow the fungi was that developed by Ashour (1954). For the preparation of enzymes the spores from each of the species were separately collected from their cultures by repeated floodings with sterile double glass-distilled water and centrifugations. 4 ml. of suspension thus

made, having spore content roughly to the order of 4×10^5 , were utilized to inoculate each flask containing 21 ml. of the medium, and incubated at 28° C. for 6 days, when a thin mat of young mycelium appeared. The contents of the flasks were then centrifuged at 5° C. at 3,000 rpm. for 30 minutes and the supernatants, stored with a layer of toluene in deep-freeze at -20° C., served as the extracellular enzyme extract.

The fungal mycelia, washed free of adhering media, were partially dried between folds of sterilized filter-papers and finally in a vacuum desiccator under vacuum and over calcium chloride. The dried mycelia were mixed with an equal quantity of acid-washed sand and crushed in glass pestles and mortars to yield fine powders, which were stored under dry conditions in deep-freeze.

For the extraction of enzymes, 0.2 gm. of the powder containing 0.1 gm. of the fungus material and 0.1 gm. of sand were suspended in 10 ml. of water or buffer at 4° C. with periodical shaking. After 16 hours the solutions were centrifuged at 3,500 rpm. for 20 minutes at 5° C., and the clear supernatants were directly used as the intracellular enzyme source. The presence of the enzymes in the intra- and extracellular extracts was tested by the usual procedures (the actual methods followed in each case have been referred to in appropriate places). Two types of controls, one with inactive enzyme (inactivated by autoclaving at 15 lb. for 15 minutes) and the other with sterile distilled water, were prepared for comparison with the tests. The results, where the enzymes have been grouped following the system adopted by Colowick and Kaplan (1955), are given in Table I.

The table shows the presence of a large number of enzyme systems and records for the first time the presence of phosphorylase, aldolase, dehydrogenases, amidases, deaminases, ribonuclease, nucleodeaminases, glycerophosphatase and

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TABLE I
Qualitative demonstration of enzymes

Enzyme	Substrate	Incubation time and temperature	Enzyme activity						Method
			Intracellular			Extracellular			
			AT	AB	AR	AT	AB	AR	
I Carbohydrate metabolism									
1 Amylase	.. Starch (5%)	24 hrs. at 37° C.	+	+	+	+	+	+	Fehling's solution do. do. do.
2 Invertase	.. Sucrose (1%)	do.	+	+	+	+	+	+	
3 Raffinase	.. Raffinase (1%)	do.	+	+	+	+	+	+	
4 Hemicellulase	.. Hemicellulose (Nutman, 1929) (1%)	do.	+	+	+	+	+	+	
5 Maltase	.. Maltose (1%)	do.	+	+	+	+	+	+	Barfoed's reagent Maceration do.
6 Cellulase	.. Filter-paper	15 days at 37° C.	+	+	+	+	+	+	
7 Protopectinase	.. Potato discs	½-20 hrs. at 37° C.	+	+	+	+	+	+	Sumner <i>et al.</i> (1944, 1950) Sibley and Lehnin- ger (1949)
8 Phosphorylase	.. Glucose 1-phos- phate (0.5%)	1 hr. at 37° C.	+	+	+	Not done			
9 Aldolase	.. Fructose-1 : 6-di- phosphate (M/20)	do.	+	+	+	do.			
II Lipid metabolism									
1 Lipase	.. Olive oil emulsion	7 days at 37° C.	+	+	+	+	+	+	Titration do.
2 Butyrase	.. Ethyl acetate (2%)	3 days at 37° C.	+	+	+	+	+	+	
III Citric acid cycle									
1 Dehydrogenases	.. Succinic acid	16 hrs. at 37° C.	+	+	+	Not done			Srikantan and Murti (1955) do.
	Aspartic acid	do.	+	+	+	do.			
IV Protein metabolism									
1 Proteolytic enzyme	.. Peptone (2%)	24 hrs. at 37° C.	+	+	+	+	+	+	Formal titration Medium around the fungal colonies became cleared
2 Erepsin	.. Casein (1%)	7 days at 28° C.	Not done			+	+	+	
3 Rennatase	.. Milk (fresh)	24 hrs. at 37° C.	+	+	+	+	+	+	Coagulation Ammonia aerated off and estimated by titration
4 Urease	.. Urea (1%)	7 days at 37° C.	+	+	+	+	+	+	
5 Amidases and Dea- minases	l-glutamic acid (M/100)	90 mts. at 37° C.	+	+	+	Not done			Liberated ammonia estimated by nesslerization do. do. do. do. do. do. do. do.
	dl-glutamic acid (M/50)	do.	+	+	+	do.			
	l-glutamine (M/100)	do.	+	+	+	do.			
	l-histidine (M/100)	do.	+	+	+	do.			
	dl-histidine (M/50)	do.	+	+	+	do.			
	d-histidine (M/100)	do.	+	+	+	do.			
	dl-asparagine (M/50)	do.	+	+	+	do.			
	dl aspartic acid (M/50)	do.	+	+	+	do.			
	l-Asparagine (M/100)	do.	+	+	+	do.			
V Nucleic acid metabolism									
1 Ribonuclease	.. Yeast RNA (4 mg./ml.)	2 hrs. at 37° C.	+	+	+	do.			Formation of pen- toses estimated by Brown's (1946) orcinol reaction Nesslerization
2 Nucleodeaminases	Adenine (M/100)	do.	+	+	+	do.			
	Guanine (M/100)	do.	+	+	+	do.			
	Xanthine (M/100)	do.	+	+	+	do.			
	Uracil (M/100)	do.	-	-	-	do.			
	AMP (M) (M/100)	do.	+	+	+	do.			
	AMP (Y) (M/100)	do.	+	+	+	do.			
	GMP (M/100)	do.	+	+	+	do.			
	CMP (M/100)	do.	+	+	+	do.			
	ATP (M/100)	do.	+	+	+	do.			

(TABLE-I—Contd.)

TABLE I—Contd.

Enzyme	Substrate	Incubation and temperature	Enzyme activity						Method		
			Intracellular			Extracellular					
			AT	AB	AR	AT	AB	AR			
VI	<i>Phosphate metabolism</i>										
1	Glycerophosphatase	Na-β-glycerophosphate (0.5%)	1 hr. at 37° C.	+	+	+	Not done			Phosphorus estimated (Sumner, 1944)	
2	Nucleophosphatases	AMP (M) (M/100)	2 hrs. at 37° C.	+	+	+	do.				do.
		ATP (M/100)	do.	+	+	+	do.				do.
		GMP (M/100)	do.	+	+	+	do.				do.
		CMP (M/100)	do.	+	+	+	do.			do.	
VII	<i>Respiratory enzymes</i>										
1	Catalase	Hydrogen peroxide (1%)	2 hrs. at 15° C.	+	+	+	+	+	+	Titration by KMnO ₄	
2	Oxidases and peroxidases	Tincture of guai-cum (2.5%) and H ₂ O ₂	4-24 hrs. at 37° C.	+	+	+	+	+	+	Colour change	
3	Laccase	.. Hydroquinone (1%)	16 hrs. at 37° C.	+	+	+	+	+	+	do.	
4	Tyrosinase	.. Tyrosine (saturated solution)	48 hrs. at 37° C.	-	-	-	-	-	-	do.	

+ denotes the presence of enzyme; - denotes the absence of enzyme.

nucleophosphatases, using various substrates, in the three species of *Alternaria* studied. Rennatase, laccase, oxidase and peroxidase, earlier reported to be absent in the enzyme preparations of *A. tenuis* (Tandon and Srivastava, 1949), were found to be present in significant quantities in the present investigations. These qualitative studies showing significant activities of various enzyme systems give encouraging possibilities for quantitative investigations with regard to the distribution and kinetics of these enzymes in the species of *Alternaria*, which in turn may lead to a possible correlation between the enzyme activity and pathogenicity of these organisms possessing a wide host range. The results of such studies will be published soon.

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